Chapter 12
Development and Regulation of the *Plum Pox Virus* Resistant Transgenic Plum 'HoneySweet'

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Abstract Genetic engineering (GE) can target specific genetic improvements and allow for the development of novel, useful traits. In spite of the potential utility of GE for fruit tree improvement, the technology has not, to date, been widely exploited for variety development due, in part, to the reticence of researchers to become involved in the regulatory process. Over the past 20 years an intensive international research project focused on the development of GE resistance to *Plum pox virus* (PPV) the causative agent of Sharka, one of the most destructive diseases of plum and other stone fruits. This effort resulted in the development of 'HoneySweet' plum, a GE variety that has proven to be highly resistant to PPV, as demonstrated in over 15 years of field testing in the U.S. and Europe. In order to make this variety available to breeders and growers in the U.S., dossiers were submitted to the U.S. regulatory agencies. This process ultimately led to the regulatory approval of 'HoneySweet' in the U.S. The work with 'HoneySweet' demonstrates that the regulatory process, while a significant effort, can be successfully navigated by public institution researchers. Nevertheless, the few examples of such success demonstrate a need for public institutions to find ways to encourage, support and reward researchers who pursue deregulation efforts. The long-standing successes of virus control in

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squash and papaya, and the current work with plum demonstrate the power and the safety of GE for specialty crop improvement. The commitment of researchers, institutional support, clear, science-based regulatory frameworks that build upon a developing knowledge base, industry support, and public outreach are components that are now necessary to move this technology forward to improve agricultural production and its sustainability.

Keywords *Prunus* • Plum • *Plum Pox Virus* • Virus resistance • Genetic engineering • Sharka • Gene silencing • Rosaceae • GE regulations • APHIS • EPA • FDA

12.1 Introduction

Genetically engineered (GE) genotypes now account for the greater part of the world acreage of some of the most widely grown and traded crops such as soybean, maize, cotton, and canola. In the West, the research and development of these GE crop varieties have been virtually the exclusive domain of large multinational corporations. These corporations have the financial resources not only to run extensive molecular research programs and breeding trials but they can also heavily invest in intellectual property (IP) issues and most importantly, they can invest the significant resources necessary for regulatory approvals, in most cases in multiple countries. The payback on these investments comes from crops with significant world-wide production. Typically, specialty crops are high value per unit land area but they are produced on relatively small land areas and are made up of a multitude of genotypes specific to particular regions and/or markets. If the production of GE varieties of specialty crops is to move forward it will likely be through the work of public institutions. The need for the use of GE technologies for the improvement of specialty crops is great. As a whole, these crops produce high incomes for growers, contribute significantly to local and regional economies, and are important components of a healthy diet. But public institutions suffer from limited funding, and industries for each specialty crop are relatively small and so cannot provide the funding necessary for robust programs that will take a GE crop variety from proof of concept to product. Public research institutions also suffer from limited experience and limited staff that can be devoted to IP and regulatory issues. University researchers are not rewarded for time spent on IP and regulatory work but instead are awarded tenure and grants for novel research that then may be taken to the stage of proof-of-concept. Research in model plants demonstrating the expression of novel transgenes with potential for crop improvement generally ends with publication but without a commercial product. Such findings may be the starting point for private enterprise to enter, taking the proof-of-concept to product, as seen with the major row crops. Unfortunately, this has generally not occurred with specialty crops for several reasons, including freedom to operate issues, and the time, costs, and uncertainties associated with regulatory approvals. The uncertainty of consumer acceptance also figures largely in the decision process of private enterprise.

The difficulties encountered in the path from proof-of-concept to GE specialty crop marketing are significant and they are real. World-wide there are only nine specialty crops in which a GE variety has been marketed or taken to the point where it can be marketed; these are tomato, potato, squash, sweet corn, papaya, flax, tobacco, carnation, and plum. This chapter will focus on the development, testing, and regulatory approval of 'HoneySweet' plum, genetically engineered for resistance to Plum pox virus (PPV), to illustrate the path from research to product taken by a public institution, the United States Department of Agriculture (USDA), Agricultural Research Service (ARS).

12.2 Background for the GE Approach

Sharka disease caused by Plum pox virus (PPV) is considered to be one of the most serious threats to stone fruit production world-wide (Cambra et al. 2006). Symptoms include fruit deformation, pitting and gumming of fruit flesh, premature fruit drop, leaf chlorosis, and in highly susceptible varieties, tree decline. Almost all species of the genus *Prunus* are susceptible (Damsteegt et al. 2006). Since its first description in Bulgaria (Atanassov 1932), the virus has spread to a large part of the European continent, around the Mediterranean basin and Near and Middle East, South and North America (Argentina, Canada, Chile, and USA) and Asia (China, Kazakhstan and Pakistan) (Cambra et al. 2006; various authors 2006) (Fig. 12.1). Long distance dispersion of the virus is through infected budwood and rootstocks. Local spread is by aphids. In order to restrict the spread of PPV the European Plant Pathology Organization (EPPO) recommends measures such as quarantine isolation, nursery

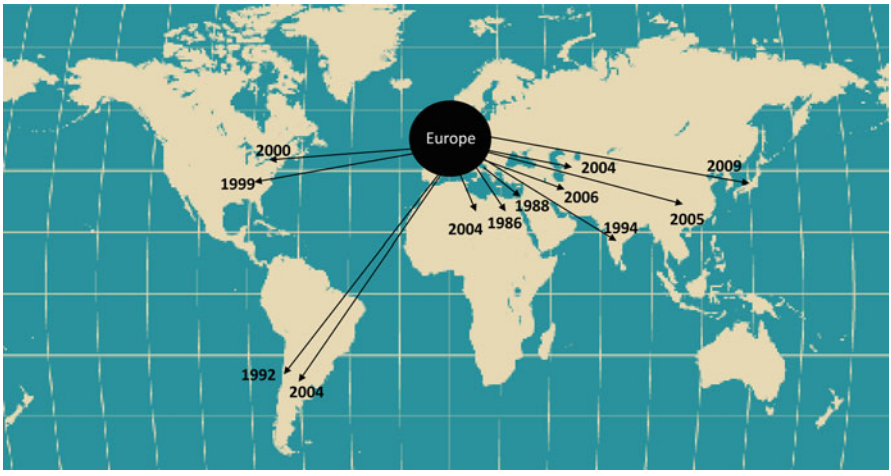

Fig. 12.1 Spread of Plum pox virus following its identification in Europe (Bulgaria) in 1918 (Atanassov 1932)

and orchard surveys, propagation of virus-free *Prunus* and chemical treatment of trees against aphid vectors. These measures have been ineffective in halting the spread of PPV which is now endemic in many European countries. Due to the rapid spread of PPV by aphids and the presence of many potential hosts, Sharka disease is difficult to eradicate once it has become established. Tree removal is the only strategy that can be used to eradicate the virus from an area. While the control of PPV through host resistance represents a preferred strategy there are few sources of high level resistance and therefore stonefruits, in general, are highly vulnerable.

12.3 Research Approach

Facing the threat of the introduction of PPV into the U.S., USDA-ARS began a program of pre-emptive breeding for PPV resistance. In 1989 researchers at the ARS- Appalachian Fruit Research Station (AFRS), Kearneysville, West Virginia began work on the development of resistance to PPV through genetic engineering. Our first studies utilized the papaya ringspot virus (PRV) coat protein (CP) gene (kindly provided by Dr. Dennis Gonsalves, Cornell University, Geneva, NY; currently USDA-ARS, Hilo, HI) which was used to develop PRV resistant papayas (Gonsalves 1998). It was thought that this virus CP gene might have enough homology to the PPV-CP gene to be effective in providing resistance to PPV. At the time that this work began, virus resistance was expected to be CP-mediated (Beachy et al. 1990). The heterologous protection against PPV in plum based on PRV-CP expression was effective for several years in greenhouse tests, but after 32 months symptoms of PPV infection appeared and plants became fully infected (Scorza et al. 1995). During the time of this work in the U.S., Michel Ravelonandro (INRA, Bordeaux, France) had isolated, sequenced and cloned the PPV coat protein (CP) gene (Ravelonandro et al. 1992). In collaboration with Ravelonandro, Gonsalves, and members of Gonsalves' research group, the PPV-CP gene was engineered into the plasmid pGA482GG (Fitch et al. 1990; Ling et al. 1991), the same plasmid that was used for the successful engineering of papaya ringspot virus resistant papayas (Fitch et al. 1992). *Agrobacterium*-mediated transformation of plum was based on the procedure developed by Mante et al. (1991) utilizing hypocotyl slices from seed derived from open pollination. The first 2 years of the project were dedicated to vector construction and testing in tobacco, transformation of plum, tissue culture of putative GE plants (selection, proliferation, rooting), greenhouse acclimation and plant propagation for testing. Confirmed transgenic plants were transferred under a USDA-Animal and Plant Health Inspection Service (APHIS) permit to the BSL3-P containment greenhouse at the USDA-ARS Foreign Disease and Weed Research Unit at Ft. Detrick, MD. At that time it was the only greenhouse facility in the U.S. where work with PPV was permitted. During the 3 years of these greenhouse-based inoculation and testing studies, one transgenic plum line appeared to be highly resistant to PPV. However, this line did not express PPV-CP and produced barely detectable levels of CP mRNA. Clones that did express the CP gene proved to be

susceptible (Ravelonandro et al. 1997; Scorza et al. 2001). This suggested that a mechanism other than CP-mediated protection was at work. The "C5" plum clone became the focus of research on the mechanism of resistance to PPV. From these studies, a series of papers describing the resistance in the greenhouse and field led to the demonstration of post-transcriptional gene silencing (PTGS) as the mechanism of resistance (Ravelonandro et al. 1997; Scorza et al. 2001; Hily et al. 2004, 2005). Silencing was based on the activity of a hairpin configuration that was apparently the result of a duplication and rearrangement during the insertion event. In 1993, a field trial of C5 and the other transgenic lines was planted at the AFRS in Kearneysville, WV under an APHIS permit. This field trial was developed not to test for resistance, since PPV was not present in the U.S. and we could not inoculate plants in the field, but rather to evaluate the trees for transgene expression and for their horticultural traits including growth habit, and fruit yield and quality. While the C5 clone appeared to be highly resistant in greenhouse tests, field testing under artificial inoculation and natural aphid-vectored disease pressure was necessary to evaluate resistance on mature trees under typical orchard conditions and in different plum-growing environments, and with different PPV strains. Collaborations were developed with research partners in Europe (T. Malinowski, Poland; I. Zagrai, Romania; and M. Cambra, Spain) to test this resistant clone in areas where PPV was established. Appropriate field test permits were granted in each country and field trials were initiated in 1996–1997, which was 6–7 years following the initial plum transformations. By 2002 the field tests clearly demonstrated the resistance of C5 to PPV infection through aphid vectors and by graft inoculation (Hily et al. 2004). Continuation of these tests through 2005 confirmed the resistance (Malinowski et al. 2006).

In December 1999, PPV was detected in peach and plum trees in orchards in Adams County, Pennsylvania (Levy et al. 2000). This detection resulted in what was to become a 10-year eradication program that cost over $65 M and resulted in almost the complete elimination of stone fruits in the affected counties. At that same time 'HoneySweet', the variety name for C5, was demonstrating an extremely high level of resistance to PPV in the European field trials. C5 trees exposed to natural aphid vectors were never found to be infected, and graft-inoculated trees showed only low virus titer near the point of graft inoculation. With the detection of PPV in the U.S., the need for resistant germplasm for U.S. growers was clear and it was decided to make 'HoneySweet' available for U.S. breeders and growers.

12.4 The Regulatory Process

The commercial availability of 'HoneySweet' required regulatory approvals from APHIS, and the U.S. Environmental Protection Agency (EPA). A voluntary submission to the U.S. Food and Drug Administration (FDA) is also typically a part of the regulatory process for GE food products. With the anticipation of regulatory submissions, risk assessment studies were initiated both in the U.S. and in Europe.

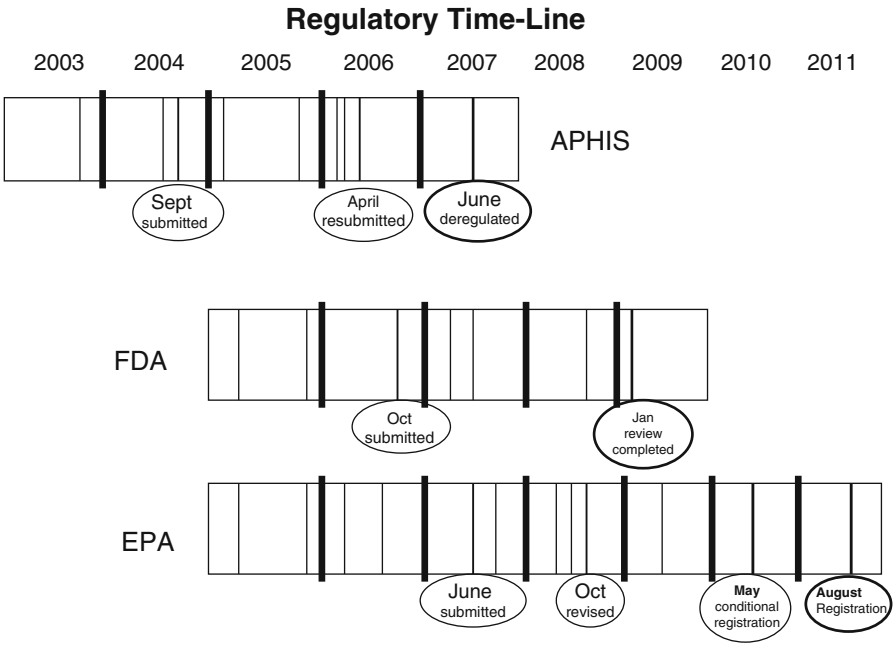

Fig. 12.2 Schedule of regulatory consultations (*thin lines*), submissions and approvals for 'HoneySweet' plum. Thin vertical lines indicate dates of meetings between regulators and applicant

Pre-submission consultations with U.S. regulatory agencies APHIS, FDA and EPA began in 2003 (Fig. 12.2). APHIS has jurisdiction over the field testing of genetically engineered plants that contain plant pathogen genes or promoters. FDA has jurisdiction over GE plants used as food, and EPA regulates GE crop plantings of over ten acres for GE plants that produce molecules that protect plants against pests - protection against PPV in the case of 'HoneySweet'. Based upon the guidance provided in these consultations, data from over 13 years of work with 'HoneySweet' in the laboratory, greenhouse and field, in the U.S. and in Europe, including risk assessment studies, were incorporated into dossiers for the regulatory agencies. An application for determination of non-regulatory status was submitted to APHIS in September 20, 2004. In February 2005 a notification from APHIS was received detailing deficiencies and clarifications that needed to be addressed in the application. The revised application was resubmitted on March 13, 2006 and deemed to be complete and accepted for review on April 7, 2006. At that time APHIS initiated, as part of its standard procedure, an Environmental Assessment (EA). In May, 2006, the petition submitted to APHIS was posted on the internet for 60 days of public comment. APHIS received 1,725 comments, 1,708 were not in support of deregulation. Many if not most comments of non-support appeared to be duplicates, cut and pasted from a single anti-GMO website. APHIS addressed the comments and a

determination of non regulated status was made on June 27, 2007. The result of the EA was a Finding of No Significant Impact (FONSI).

The dossier provided to the FDA consisted of information pertaining to the food uses of plum, and compositional analyses of 'HoneySweet' and control, untransformed plums. To obtain this data fruit samples from several varieties of plum of similar age and located near the 'HoneySweet' planting were collected and sent to a commercial laboratory for analysis. Information pertaining to allergenicity and antinutrients was obtained through the collaboration of ARS colleagues at the USDA-ARS- Eastern Regional Research Center, Wyndmoor, PA. The purpose of the analyses was to determine if any transgene sequences would be predicted to produce proteins that matched known allergenic or anti-nutrient proteins. Several databases and alignment approaches were used including the Allermatch allergen finder (www.allermatch.org), 7 and 8 amino acid word search using the same database, 80 amino acids sliding window alignment with the same database, and FASTA alignments done manually using the Codex Alimentarius guidelines which were used to create the Allermatch algorithms. The sequence was broken into 80 amino acid words and FASTA aligned with allergens (http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf).

The antinutrient potential of the insert sequences was evaluated using the NCBI antinutirent sequence data base. The submission to FDA was made on October 26, 2006 and was accepted on January 12, 2007. Additional information and\or clarifications were provided at the request of FDA on April 5, June 3, June 12, 2007 and on September 19, 2008. A final letter of "no further questions" was received from FDA on January 16, 2009. In the language typical of such a letter, the FDA stated that, "Based on the safety and nutritional assessment USDA-ARS conducted, it is the understanding of FDA that USDA-ARS has concluded that plums derived from the new variety are not materially different in composition, safety, and other relevant parameters from plums currently on the market and that the genetically engineered plum line C5 does not raise issues that would require premarket review of, or approval by, FDA."

Although 'HoneySweet' produced no PPV-CP and although PPV-infected plums – which are widely consumed in Europe-- contain PPV-CP, EPA determined that the PPV-CP gene in 'HoneySweet' plum would be considered as a plant incorporated protectant (PIP) and that 'HoneySweet' should be regulated and registered as a biopesticide. The format for EPA registration of a biopesticide is administratively complex. In order to expedite the submission process and allow the researchers to focus on putting together the necessary scientific documentation rather than working on the administrative issues of the EPA regulatory process, ARS sought the assistance of the Interregional Research Project Number 4 (IR-4), an organization that functions to submit minor use pesticide registration packages and tolerance petition applications to EPA. IR-4 assumed the responsibility of taking the data provided by ARS researchers and developing a submission package that conformed to the formatting requirements of EPA. The dossier was submitted in June, 2007. The submission included a Registration Volume of administrative materials and four additional volumes consisting of Volume 1- Tolerance Exemption petition for the PPV resistance gene (the PPV-CP gene); Volume 2, Product Chemistry of the PPV

Resistance Gene; Volume 3 PPV - Resistance Gene Non-target Waiver Requests; and Volume 4 - PPV Resistance Gene Health Waiver Requests. The submission was found to be in compliance with the data submission standards contained in Pesticide Registration (PR) Notice 86-5 (see http://www.epa.gov/PR_Notices/pr86-5.html). During the review period EPA made several requests to the ARS submitter for conformance to EPA documentation guidelines and clarification of information and submission of additional information. Each request "stopped the clock" on the review process, adding additional time to the EPA review process. The initial scientific review resulted in a September 2007 request for additional information. This request required clarification of figures, additional bioinformatic analyses, and clarification of bioinformatic analyses that had been submitted. EPA required sequence-based analyses of toxicity, allergenicity, and antinutrient potential of the PPV-CP and associated transgenes based on similarity to sequences known to exhibit these properties, and an individual volume addressing these issues was submitted. Under regulation (40 CFR 152.105) (http://cfr.vlex.com/vid/152-105-incomplete-applications-19815353), EPA is obliged to allow 75 days to address the deficiencies in the application. The level of analyses required to comply with the EPA request for additional information made it necessary that we request an extension of the Pesticide Registration Improvement Renewal Act (PRIA) due date (the date that EPA would complete the registration decision) which EPA granted. While the September 2007 request for additional information was being addressed, another request for additional information was received from EPA in February 2008. During this period meetings with EPA were held in order to clarify the requests and to discuss issues including the propagation, production and distribution of fruit trees, tree labeling and associated horticultural issues. Responses to the information requests of September 2007 and February 2008 along with hard copies of all cited references in the original submission and supplemental submissions were submitted to EPA in July 2008. On October 29, 2008 EPA published in the Federal Register (73 FR 64325) a Notice of Receipt announcing that IR-4 submitted on behalf of the USDA-ARS-AFRS (the applicant) an application to register a pesticide product containing a new active ingredient not included in any currently registered pesticide product (the PPCV-CP gene). Four comments were received during a 30 day comment period following the publication of the notice, all favorable. A petition (7E7231) seeking an exemption from the requirement of a tolerance for residues of the PPV-CP in stone fruit and almonds was filed by IR-4 on behalf of the UDSA-ARS-AFRS. EPA published a notice of filing of the petition in the Federal Register on November 14, 2008 (73 FR 67512) and the public was given a 30 day comment period. EPA received no comments on this notice. During the EPA review process we requested a number of conference calls and face-to-face meetings with EPA in order to obtain information on the status of the review and the status of the requested exemption of tolerance for the PPV-CP in stone fruits and almond. These meetings helped us to provide information to EPA that was relevant to their decision-making process. EPA informed us that an independent laboratory validation (ILV) of our proposed method for detecting the transgene in 'HoneySweet' leaves would be required and we began the process of soliciting a laboratory that the EPA considered appropriate. In

December 2009, EPA indicated a need to extend the PRIA date from January 8, 2010 to July 8, 2010. The need for this extension was the result of a new transparency requirement initiated by EPA which required a 30 day public comment period on the draft registration decision followed by a 60 day period during which the public would have the opportunity of submitting objections or hearing requests. The 'HoneySweet' petition although well underway and very close to a final decision, was not grandfathered-in but was subject to the process. Due to this new requirement and the need for EPA to review the draft ILV protocol, EPA proposed a 6 month PRIA extension. A 4 month (May 8, 2010) PRIA extension was negotiated. On April 1, 2010 the draft registration was published on the web (http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2008-0742) with a comment period ending on April 30, 2010. Seventy eight comments were received; seventy six were highly supportive of registration, including some eloquently questioning the need for registration and the classification of 'HoneySweet plum as a biopesticide. Comments included opinions that the mechanism of resistance does not produce a PIP since no CP is produced and DNA has never been considered alone to be a pesticidal substance. The labeling of trees as pesticidal was also brought into question. It was suggested that mandatory labeling of 'HoneySweet' trees and propagative material as pesticidal (fruit would not be labeled) would cause substantial damage to the market for 'HoneySweet' and sets a precedent for future transgenic virus resistant crops to be treated "in the same unscientific and irrational manner." (for specific comments cited see http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2008-0742). On May 7, 2010 EPA issued a 1 year conditional registration for 'HoneySweet' plum. A major condition of the registration to be fulfilled within 1 year was the ILV. At the time of conditional registration EPA agreed on the methodology in the protocol and the selection of the independent laboratory (Field Laboratory Services, Agricultural Marketing Service, Gastonia, NC) but the validation had not yet been performed. On November 2, 2010 the completed ILV was received by EPA and it was approved on January 13, 2011. The unconditional Sect. 12.3 registration was issued on August 8, 2011.

A final rule establishing the exemption from tolerance was effective on May 26, 2010 EPA-HQ-OPP-2008-0763; FRL-8826-9 (http://edocket.access.gpo.gov/2010/2010-12579.htm). This exemption clears the future use of PPV-CP genes for genetically engineered resistance to PPV in stone fruits and almonds whether the CP is expressed or not, without the necessity of seeking a tolerance level for PPV-CP.

12.5 Conclusions

At the time of this writing PPV continues to elude eradication efforts in Canada and is slowly spreading in New York State. Although federal and state authorities are working to prevent disease spread through culling and quarantine programs the multi-state detection of PPV clearly indicates that U.S. growers remain at risk from future PPV outbreaks. California produces 99 % of the U.S. plum supply and

40–60 % of the world supply of dried plums (prunes). The export value is $132 M. PPV presents a serious threat to this industry. The history of PPV spread world-wide demonstrates that conventional control methods such as chemical control of insects, quarantine, and even eradication efforts have proven to be costly and, in the long-term, unsuccessful. Disease-resistant fruit trees would provide the U.S. industry with a long-term, sustainable solution to the threat of PPV spread and would help to prevent the spread of PPV into susceptible native *Prunus* species which are virtually all susceptible (Damsteegt et al. 2006). There are few reports of naturally occurring high level, multi-strain resistance to PPV in most commercial *Prunus* species. Resistance has been reported in apricot (Ruiz et al. 2011) and hypersensitivity has been reported in plum (Hartmann and Petruschke 2002) and this mechanism can provide a reasonable level of resistance in plum if properly managed (Polák et al. 2005). We have demonstrated that genetic engineering can be an important source of high level and durable resistance against all known strains tested thus far. We have shown through a number of field studies the environmental safety of this technology (Capote et al. 2008; Fuchs et al. 2007; Zagrai et al. 2008, 2011). Nevertheless, the utilization of this demonstrated effective technology for the practical control of PPV has not occurred outside of the work with 'HoneySweet'. There are a number of reasons for this situation as discussed in the introduction to this chapter and elsewhere in this book. Clearly, the reticence of researchers to become involved in the regulatory arena is among these. Institutions supporting agricultural research need to find ways to encourage, support, and reward researchers who pursue regulatory approval efforts. The IR-4 Project (http://ir4.rutgers.edu/), represents a pathway for registration to public sector researchers and is currently assisting in the registration of other transgenic crops. Other organizations such as the Public-Sector Intellectual Property Resource for Agriculture (PIPRA) (http://www.pipra.org/) and Specialty Crop Regulatory Assistance (SCRA) (http://www.specialtycropassistance.org/) are also available to assist in navigating intellectual property and regulatory issues. When feasible, industry partners should be sought that have an interest in bringing a potential product through the regulatory process. Regulations should be science-based with clear submission criteria and should seek to minimize the cost and bureaucracy associated with submissions. The long-standing successes of virus control in squash and papaya (Oliver et al. 2011) and the current work with plum demonstrate the power and the safety of this approach. Institutional support, the commitment of researchers, clear, science-based regulatory frameworks that build upon a developing knowledge base, industry support, and public outreach are components that are now necessary to move this technology forward to improve agricultural production and its sustainability.

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